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REMARKS

Telephonic interview statement (MPEP 713.04). The instant Response and Amendment is accompanied by a RCE, which, as summarized in the Examiner's telephonic "Interview Summary" of 26 March 2004, is being filed by the applicants "in response to the final rejection for the purpose of having a supplementary IDS considered." Applicants thank the Examiner for the telephonic interview and for authorizing submission of the IDS without paper copies of those listed citations corresponding to U.S. Patents, U.S. applications and WO publications, unless requested by the Examiner to do provide them.

Claims 1-3, 8-10, 18-20 and 38-41 are pending. Claims 4-7, 11-17, and 21-37 were previously cancelled, having been withdrawn by the Examiner pursuant to 37 C.F.R. 1.142 § (b).

Applicants thank the Examiner for *withdrawal* of the following objections and rejections in view of applicants' last Response and Amendments, including the clarifying amendments to exclude SEQ ID NOs:11 and 12, and contiguous fragments thereof from the claimed subject matter:

- objection to the specification in view of alleged embedded hyperlinks, and in view of improper dependency of claims 9 and 10 (*see* Office action of 21 October 2003, page 2, para 3);

- objection relating to final formal drawings (*Id*, at page 2, para 4);

- rejection of claims 1-3, 9, 10 and 18 under U.S.C. § 112 ¶2 (*Id*, at page 2, para 5);

- rejection of claims 1-3, 8-10 and 18-20, under 35 U.S.C. § 102(e), in view of Doherty et al (U.S. Patent No. 6,414,130) (*Id*, at pages 3-4, paras 6-8);

- rejection of claims 1-3, and 8-10, under 35 U.S.C. § 102(a), in view of Doherty et al (Doherty et al., *Proc. Nat. Acad. Sci.*, USA 96(19):10869-10874, 1999, Sept.) (*Id*, at pages 3-4, paras 6-8);

- provisional rejection of claims 1-3, 8-10 and 18-20 under the judicially created doctrine of obviousness-type double patenting in view of applicants' copending U.S. Application Serial No. 09/234,208 (*Id*, at page 4, para 9);

- rejection of claims 1, 3, 18 and 19, under 35 U.S.C. § 102(b), as being anticipated by Greene et al (U.S. Patent No. 5,464,751; issued 7 Nov. 1995) (*Id*, at page 4, para 10);

-rejection of claims 1, 3, 18 and 19, under 35 U.S.C. § 102(b), as being anticipated by Wels et al (U.S. Patent No. 5,571,894; issued 5 Nov. 1996) (*Id*, at page 4, para 11); and

-rejection of claims 1, 3, 18 and 19, under 35 U.S.C. § 102(e), as being anticipated by Ring (U.S. Patent No. 6,054,561; issued 25 April 2000, filed 7 June 1995) (*Id*, at page 4, para 12).

Applicants acknowledge that the Examiner has, in view of applicants' last amendments (*i.e.*, excluding SEQ ID NOS:11 and 12, etc. from the claimed subject matter), *maintained* the rejection of claims 1-3, 8-10, and 18-20, under 35 U.S.C. 112 ¶1 (alleging lack of written description), has extended this rejection to claims 38-41, and has asserted an allegation of *new matter* as a *new ground* of rejection for all of these claims (*Id*, at page 5, para 13). Applicants respectfully traverse these grounds of rejection as discussed in detail herein below.

Applicants also acknowledge that the Examiner has *maintained* the rejection of *independent* claim 18, under 35 U.S.C. § 102(e), as being anticipated by Hudziak (U.S. Patent No. 6,399,063) (*Id*, at pages 5-6, para 14). Applicants have amended claim 18 herein below to bring it into allowable form, based on the Examiner's comments.

Applicants have further amended the Sequence Listing, attached hereto as **APPENDIX D**, to include two (2) additional SEQ ID NOS:14 and 15 to allow for proper claiming of these species of originally filed SEQ ID NO:1 and SEQ ID NO:2, respectively. SEQ ID NOS:14 and 15 correspond the most common amino acid sequence of applicant's Figure 8.

Finally, applicants have added new species claims 42-49 drawn to isolated polypeptides corresponding to SEQ ID NO:14 (claims 42-45) and SEQ ID NO:15 (claims 46-49), which are species of SEQ ID NOS:1 and 2, respectively.

No new matter has been added.

FORMALITIES

Applicants have, pursuant to MPEP 201.11, deleted the priority claim to U.S. Patent Application Serial No.09/234,208.

Applicants have previously submitted final formal drawings in response to the Draftsperson's comments.

Applicants have non-substantively amended *dependent* claim 9 to delete the word "and" and to insert the words "at least," to correct grammar and conform with the language, respectively, of *independent* claim 8. No new matter has been added.

Rejections under 35 U.S.C. § 112 ¶1 (written description / new matter)

The Examiner maintained the rejection of claims 1-3, 8-10 and 18-20 under 35 U.S.C. § 112 ¶1, alleging that the Specification was lacking adequate written description to "reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention" (*see* Office Action of 21 October, at page 5, para 13). This rejection was extended to added claims 38-41 (*Id*).

Specifically, the Examiner asserts that the "disclosure of the specification fails to describe the *subgenus* of compounds encompassed by the claims," as amended by applicants to exclude the prior art sequences (applicants' own) SEQ ID NO:11 or SEQ ID NO:12, or a fragment of SEQ ID NO:11 that is 50-79 contiguous residues in length, or a fragment of SEQ ID NO:12 that is 80-419 contiguous residues in length." The Examiner alleges that in the absence of support for the subgenus, the prior "amendment to the claims and the addition of new claims 38-41 [that also exclude SEQ ID NO:11 or SEQ ID NO:12, etc.] constitutes new matter" (*Id*).

Applicants respectfully *traverse* this rejection, based on the fact the originally filed Specification has clear and sufficient support/written description for the novel polymorphisms (subgenus) claimed in the currently amended claims. Specifically, support for recitation of the presently claimed subgenus is found, *inter alia*, in Example 11 and Table 1 of Example 11 of the Specification at pages 32 and 33.

As an initial matter, Example 11 and the "additionally" (apart and distinct from the prior art) disclosed novel polymorphisms thereof, is in fact the *essential* reason/motivation for filing the instant application in the first place (*see* Declaration of Dr. Gail Clinton, attached hereto as **APPENDIX A**) (*see also* Example 11, attached hereto as **APPENDIX B** to facilitate the present

analysis). Simply stated, the instant patent application describes and claims the novel polymorphisms discovered in the intron-8 encoded portion of Herstatin.

Table 1 of Example 11 is entitled “Sequence variants in the intron-8 encoded domain found in the human population (based on 15 different individuals).” Significantly, Example 11 and Table 1 *explicitly* and *unambiguously* set out and describe the presently claimed novel variants, *i.e.*, the subgenus of Herstatin variants.

In Example 11, DNA from 15 individuals was sequenced and the intron-encoded sequence *polymorphisms* were listed in Table 1 as “variants” 1-11. The term “variant,” as used in this context, is defined in relation to the most common sequence found in this region, the nucleotide sequence of Figure 8, and not with respect to SEQ ID NO:11 (which is identical to applicant’s prior art sequence) (*see* Specification at page 32, lines 10-11). Not surprisingly, applicants’ own prior art Herstatin sequence occurs as one of the sequenced polymorphisms, and is listed in Table 1 as “variant 11.” Thus, applicants’ prior art polymorphism is a ‘variant’ in the sense that its sequence varies from that of the most common sequence of Figure 8.

Significantly, however, it does not follow that simply because Example 11, and Table 1 specifically, included variant 11(prior art form), that the entire Example, as a whole, fails to explicitly describe and distinguish the novel additional polymorphisms from the prior art variant 11 (SEQ ID NOs:11 and 12, etc.). Table 1 merely serves as an efficient way to summarize all the polymorphisms (prior art and additional) by listing them.

In fact, Example 11 distinctly sets out and describes the presently claimed polymorphism subgenus. The Example begins with a description of the prior art Herstatin sequence that is in fact the instant SEQ ID NO:11 (*see* Specification at page 32 lines 1-7), *citing* Doherty et al. Proc. Natl. Acad. Sci. USA 96:10869-10874; attached hereto as **APPENDIX C**). Figure 1(A) of Doherty et al. contains the same sequence as Figure 1A of the present patent application, and Figure 1(A) of the parent application. The Example explicitly indicates that the prior art sequence of Figure 1(A) is the polymorphic form (variant 11) “that differs at amino acid residues #6 and #73 from the most commonly detected sequence shown here in Figure 8” (*see* Specification at page 32 lines 16-20). It

is thus clear that applicants were aware at the time of filing that variant 11 was already known, because it is recited in the context of Figure 1(A) and the Doherty et al reference (*Id*).

The Example then discusses the “**additionally**” (line 8) disclosed or “different” (line 11) (that is, additional with respect to, or different from the prior art SEQ ID NOs:11 and 12 , etc.) polymorphisms that were disclosed in the Example (*see* Specification at page 32 lines 8-13).

In essence, applicants’ Example 11 introduces the known prior art sequence (SEQ ID NO:11, *aka* the sequence of Figure 1(A)), and then goes on to describe and disclose that an “additional” group of polymorphisms has now been discovered. Again, the fact that the prior art polymorphism variant 11 is listed in Table 1, does not negate applicants’ discovery of polymorphisms of the inton-8 encoded portion described and disclosed in the present application at the time of filing of the presently claimed novel polymorphisms.

In fact, inclusion of SEQ ID NOS:11 and 12, etc. within the scope of applicants’ originally filed claims was inadvertent.

Applicants, in response to the Examiner’s assertion of a provisional double patenting rejection in view of the prior art sequences SEQ ID NOs:11 and 12, etc., subsequently excluded these sequences from the claimed subject matter. Significantly, however such exclusion merely serves to clarify the inventive subject matter as conceived by applicants at the time of filing, and as fully supported by the teachings and description of Example 11 of the originally filed Specification as described herein above.

In summary, applicants contend that the Examiner’s 35 U.S.C. § 112 ¶1/new matter rejection is inappropriate when applicants’ Example 11 is properly construed. Applicants, therefore, request withdrawal of this rejection in view of claims 1-3, 8-10, 18-20 and 38-41, as presented herein.

Rejections under 35 U.S.C. § 102

The Examiner has maintained the rejection of independent claim 18, under 35 U.S.C. § 102(e) as being anticipated by **Hudziak** (U.S. Patent 6,399,063; issued 04 June 2002; effective filing date of 25 January 1988) (*see* Office Action of 21 October 2003, at page 5, para 14).

Specifically, the Examiner asserts that “Hudziak discloses pharmaceutical compositions comprising an antibody to HER-2 and a second agent, such as a cytokine (TNF-alpha, TNF-beta, IL-2, Interferon-gamma; *see* col. 7, lines 3-61; claims 8-13),” allegedly thereby disclosing the instant claimed pharmaceutical compositions in view of the fact that applicants’ claim 18 “proviso language does not limit the second agent to either the agent of ‘a’ or ‘b’” (*Id.* at page 6).

Applicants have, accordingly, made a further clarifying amendment to independent claim 18, which now recites “with the proviso that where the composition comprises the monoclonal antibody it also comprises at least one of the agents of (a) or (b).”

Support for this amendment is found in the originally filed Specification at, *inter alia*, page 10-11, under “Pharmaceutical Composition.” No new matter has been added.

Applicants, therefore, respectfully request withdrawal of the Examiner’s § 102(e) rejection with respect to currently amended independent claim 18 in view of Hudziak.

New Claims

Applicants have added new claims 42-49 drawn to isolated polypeptides corresponding to SEQ ID NO:14 (claims 42-45) and SEQ ID NO:15 (claims 46-49), which are species of SEQ ID NOS:1 and 2, respectively. These species claims correspond to the most-common sequence of applicants’ originally filed Figure 8 (*i.e.*, the sequence that applicants’ Table 1 of Example 11 is based on). Support for these claims is found therefore in the originally filed SEQ ID NOS;1 and 2, Figure 8, and Table 1 of Example 11. No new matter has been added..

Applicants, to facilitate proper species claiming, have further amended the Sequence Listing, attached hereto as **APPENDIX D**, to include these two species, SEQ ID NOS:14 and 15, of originally filed SEQ ID NO:1 and SEQ ID NO:2, respectively. No new matter has been added.

CONCLUSION

In view of the foregoing amendments and remarks, applicants respectfully request entry of the present Amendment and allowance of claims 1-3 (all Previously amended), 8 (Previously

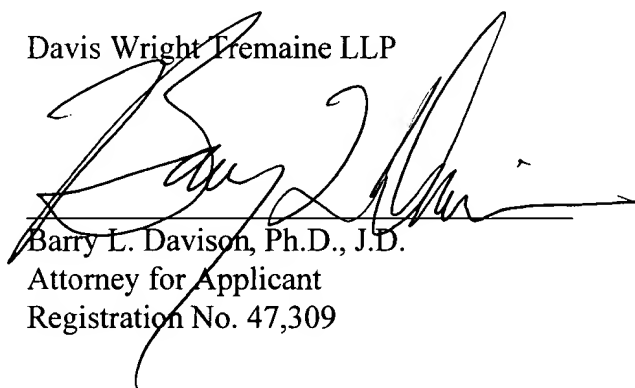
amended), 9 (Currently amended), 10 (Previously amended), 18 (Currently amended), 19-20 (Previously amended), 38-41 (Previously added), and 42-49 (New), all as provided herein above.

The Examiner is encouraged to phone applicants' attorney, Barry L. Davison, to resolve any outstanding issues and expedite allowance of this application.

No new matter has been added.

Respectfully submitted,

Davis Wright Tremaine LLP

A handwritten signature in black ink, appearing to read 'Barry L. Davison', is written over a horizontal line.

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**Example 11**

This example illustrates that the sequence of intron 8 is polymorphic. Intron 8 of the human HER-2 gene is alternatively retained in mRNA, and encodes a novel 79-residue domain at the C-terminus of a part of the extracellular domain of p185HER-2. The product, "herstatin," of the alternative transcript with the retained intron functions as an autoinhibitor of the HER-2 oncogene. The intron 8 encoded domain, alone, was shown to bind with nM affinity to p185HER-2. (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999).

Additionally, polymorphisms in the nucleotide and deduced amino acid sequence of intron 8 in the HER-2 gene were identified by sequencing genomic DNA from 15 different individuals. Figure 8 and SEQ ID NO:1 show the most common nucleotide and corresponding amino acid sequences, respectively, of intron 8. This region contains 10 different polymorphisms (marked by the letters W (2x), Y (3x), R, N, M, and S (2x) in SEQ ID NO:10; or marked by an "X" in Figure 8) that result in nonconservative amino acid substitutions (see legend to TABLE 1). For example, the polymorphism (G → C) at nucleotide position 161 (Figure 8; TABLE 1) would result in a substitution of Arginine (R) for Proline (P) at amino acid residue #54 of SEQ ID NO:1, or residue #394 of SEQ ID NO:2.. The N-terminal Glycine (G), designated as position 1 in Figure 8 or SEQ ID NO:10, corresponds to amino acid residue #341 in the "herstatin" sequence (Doherty et al., *Proc. Natl. Acad. Sci. USA* 96:10,869-10,874, 1999). The nucleotide sequence shown in Figure 1(A) is a polymorphic form that differs at amino acid residues #6 and #73 from the most commonly detected sequence shown here in Figure 8.

This result demonstrates that in the human population there are several variations in the intron-8 encoded domain that could lead to altered biochemical and biological properties among ECDIIIa-containing protein variants. Some identified variants are summarized in Table 1.

TABLE 1

	X(4)	X(14)	X(17)	X(47)	X(54)	X(62)	X(106)	X(161)	X(191)	X(217)
Variant 1	T									
Variant 2		C								
Variant 3			T							
Variant 4				A						
Variant 5					A					
Variant 6						C, T, A				
Variant 7							A			
Variant 8								G		
Variant 9									T	
Variant 10										C
Variant 11			T							C

Table 1. Sequence variants in the intron-8 encoded domain found in the human population

- 5 (based on 15 different individuals). Sequence variants 1-11 are listed, showing the base changes at a particular “X” positions relative to that found in the most common DNA sequence shown in Figure 8. The numbers in parenthesis after each X correspond to the nucleotide position in the DNA sequence shown in Figure 8. The DNA sequence variants listed here correspond to the variable amino acid positions (“Xaa”) of SEQ ID NO:1 as follows: X(4) to Xaa(2); X(14) to
- 10 Xaa(5); X(17) to Xaa(6); X(47) to Xaa(16); X(54) to Xaa(18); X(62) to Xaa(21); X(106) to Xaa(36); X(161) to Xaa(54); X(191) to Xaa(64); X(217) to Xaa(73); and to the variable amino acid positions of SEQ ID NO:2 as follows: X(4) to Xaa(342); X(14) to Xaa(345); X(17) to Xaa(346); X(47) to Xaa(356); X(54) to Xaa(358); X(62) to Xaa(361); X(106) to Xaa(376); X(161) to Xaa(394); X(191) to Xaa(404); X(217) to Xaa(413). The specific amino acid changes
- 15 (relative to the most common DNA sequence of Figure 8) for the variable amino acid positions in SEQ ID NO:1 are: Variant 1, Xaa(2)(Thr→Ser); Variant 2, Xaa(5) (Leu→Pro); Variant 3, Xaa(6) (Pro→Leu); Variant 4, Xaa(16) (Leu→Gln); Variant 5, Xaa(18) (Met→Leu); Variant 6, Xaa(21) (Gly→Asp, Alu or Val); Variant 7, Xaa(36) (Leu→Ile); Variant 8, Xaa(54) (Pro→Arg); Variant 9, Xaa(64) (Pro→Leu); Variant 10, Xaa(73) (Asp→Asn), and Variant 11, Xaa(6) (Pro→Leu) and
- 20 Xaa(73) (Asp→Asn). The same substitutions apply to the corresponding variable amino acid positions in SEQ ID NO:2.

The *HER-2/neu* receptor tyrosine kinase gene encodes a secreted autoinhibitor

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Communicated by Tony Hunter, The Salk Institute for Biological Studies, San Diego, CA, July 12, 1999 (received for review April 14, 1999)

ABSTRACT *HER-2/neu* (*erbB-2*) encodes an 185-kDa orphan receptor tyrosine kinase that is constitutively active as a dimer and displays potent oncogenic activity when overexpressed. Here we describe a secreted protein of ~68 kDa, designated herstatin, as the product of an alternative *HER-2* transcript that retains intron 8. This alternative transcript specifies 340 residues identical to subdomains I and II from the extracellular domain of p185HER-2 followed by a unique C-terminal sequence of 79 aa encoded by intron 8. The recombinant product of the alternative transcript specifically binds to *HER-2*-transfected cells with a K_D of ~14 nM and was chemically crosslinked to p185HER-2, whereas the intron encoded sequence alone also binds with high affinity to transfected cells and associates with p185 solubilized from cell extracts. The herstatin mRNA is expressed in normal human fetal kidney and liver, but is at reduced levels relative to p185HER-2 mRNA in carcinoma cells that contain an amplified *HER-2* gene. Herstatin appears to be an inhibitor of p185HER-2, because it disrupts dimers, reduces tyrosine phosphorylation of p185, and inhibits the anchorage-independent growth of transformed cells that overexpress *HER-2*.

The *HER-2/neu* (*erbB-2*) oncogene encodes a receptor-like tyrosine kinase, p185HER-2, that has been extensively investigated because of its role in several human carcinomas and in mammalian development (1–3). The function of the *HER-2* gene has been examined mainly by the structure and biochemical properties of the 185-kDa protein product of the 4.5-kb transcript (4, 5). P185HER-2 shares a common structural organization with other epidermal growth factor receptor (EGFR) family members and consists of an extracellular domain (ECD), a single transmembrane segment, and a cytoplasmic tyrosine kinase domain. Dimerization of receptor tyrosine kinases, which typically is induced by ligand binding, is required for their activation and subsequent steps in signal transduction (6). Although p185HER-2 is highly homologous to the EGFR, no ligand that directly binds with high affinity to p185 has yet been identified (1–3). Instead of activation by direct ligand binding, p185HER-2 exhibits constitutive activity in the apparent absence of ligand, which is enhanced by *HER-2* overexpression (7–10), or p185 is recruited into heterodimers by ligand-binding members of the EGFR family (11–13). Elevated basal kinase activity and constitutive dimerization as well as the status of p185HER-2 as the preferred heterodimer partner (14, 15) may contribute to its exceptional oncogenic potency.

The most common mechanism by which *HER-2* transforms cells is by overexpression of normal p185HER-2. Overexpression, with no evidence of mutations, occurs in several human adenocarcinomas (1, 2, 16). Importantly, elevated p185HER-2

in 25–30% of breast cancers predicts significantly lower survival rates and shorter time to relapse (16, 17). Moreover, systemic administration of antibodies against the ECD of p185HER-2 can increase the time to recurrence in a subset of patients with metastatic breast cancer (18).

Here we describe a secreted protein, herstatin, that exhibits high-affinity association with p185HER-2 and is encoded by the *HER-2* gene itself. Evidence is presented that herstatin is a specific inhibitor and therefore has potential importance in the regulation of p185HER-2 in normal and malignant development.

MATERIALS AND METHODS

Cell Culture. Cell lines were from the American Type Culture Collection or the Vollum Institute Core Culture Facility. Ovarian surface epithelial cell line, IOSEVAN, was provided by Karin Rodland at the Oregon Health Sciences University. NIH 3T3 parental cells and a cell line stably transfected with *HER-2*, designated 17-3-1, were a gift from Applied Biotechnology, and the NIH 3T3 cells transfected with *src*⁵²⁷ were supplied by Brian Druker of Oregon Health Sciences University. Cells were maintained in DMEM supplemented with 10% FBS, and 0.4 mg/ml of G418 (Geneticin, GIBCO/BRL) was added to the transfected 3T3 cell cultures.

Antibodies. Anti-ECDIIIa antisera were produced by Calico Biologicals (Reamstown, PA) by injection of rabbits with purified polyhistidine-tagged ECDIIIa peptide. Polyclonal anti-neu(C) was made against a peptide identical to the last 15 residues of the carboxyl terminus of p185HER-2 (19). Monoclonal antiphosphotyrosine antibody, 4G10, was a gift from Brian Druker.

PCR and Primer Sets. An SKOV-3 cDNA library (Origene Technologies, Rockville, MD) was subjected to PCR using a forward primer (A) identical to nucleotides 142–161 of *HER-2* cDNA (5'-TGAGCACCATTGGAGCTGGC-3'), which spans the initiation codon (underlined) and a reverse primer (B) (5'-TCCGGCAGAAATGCCAGGCTCC-3'), which is complementary to *HER-2* nucleotides 1265–1286 (4). Thirty cycles of 94°C for 30 sec, 58°C for 45 sec, and 68°C for 3 min were used for amplification.

Construction of Expression Vectors and Purification of ECDIIIa C-Terminal Peptide and Full-Length ECDIIIa Protein. The ECDIIIa sequence was amplified from a cDNA library and cloned into the pET30a vector, which encodes six histidine residues at the amino terminus of the expressed protein (Novagen). The His-tagged ECDIIIa protein was

Abbreviations: EGFR, epidermal growth factor receptor; ECD, extracellular domain; CM, conditioned media.

Database deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF177761).

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solubilized from bacteria, absorbed onto Ni-nitrilotriacetic acid agarose (Novagen), eluted with 250 mM imidazole, and was approximately 90% pure by Coomassie blue staining of gels.

For bacterial expression of the full-length ECDIIIa protein, a cDNA expression vector was constructed by subcloning the 1.3-kb *NcoI*-*EcoRI* fragment of *HER-2* cDNA (nucleotides 149-1456) into pET30a with an N-terminal 6× His tag, and by replacing the 160-nt *BstI*-*BglII* fragment (nucleotides 1075-1235) with the 435-nt *BstI*-*BglII* fragment containing the 274-nt ECDIIIa insert sequence cloned from a cDNA library. The BL21 strain of bacteria was doubly transformed with pET-p50ECDIIIa and with a thioredoxin expression plasmid (20), which was required to obtain a soluble form of p50ECDIIIa. Soluble extracts were applied to Ni-nitrilotriacetic acid agarose, which was washed with 20 vol of buffer containing 100 mM imidazole. The p50ECDIIIa protein was eluted with buffer containing 1.5 M imidazole, 0.5 mM NaCl, and 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and then was dialyzed against 50 mM Tris, pH 9.5 with 5 mM DTT. Treatment with 5 mM DTT for ≈ 16 hr before storage under nitrogen at -80°C was required to achieve a disulfide bonded form that migrated at a faster rate in nonreducing gels. The p50ECDIIIa protein was estimated at 70% purity in Coomassie blue-stained gels.

For mammalian expression of the full-length ECDIIIa protein, the acceptor (3') splice site was deleted by PCR using a primer spanning the initiation codon and the *NcoI* site (nucleotides 145-166 in the *HER-2* cDNA sequence) and a second primer complementary to the stop codon in the ECDIIIa sequence (nucleotides 1407-1409), which contained a *HindIII* site. The *NcoI*-*HindIII* fragment was cloned into a mammalian expression vector pJPA 5, which is driven by a cytomegalovirus promoter, (J.P.A., unpublished work).

Ligand Binding and Crosslinking Analyses. Recombinant ECDIIIa peptide, or p50ECDIIIa, purified from bacteria, were labeled with ¹²⁵I by using Bolton Hunter Reagent (ICN) to a specific activity of about 4 × 10⁴ cpm/pmol. Increasing amounts of radiolabeled protein, in the presence and absence of 100-fold excess unlabeled protein, were added in binding buffer (DMEM with 1% BSA) to about 10⁵ 17-3-1 cells or parental NIH 3T3 cells at room temperature for 1 hr. Cells were washed and extracted, and the radioactivity was quantitated.

RESULTS

A Unique 274-nt Sequence Is in *HER-2* mRNA. PCR was used to investigate *HER-2* mRNA diversity within the ECD coding sequence. A cDNA library from SKOV-3 ovarian carcinoma cells was examined by using a forward primer specific for exon 1 (21), nucleotides 142-161, and a reverse primer complementary to nucleotides 1265-1286 in exon 9 (22). A product of ≈ 1,420 nt, determined to be *HER-2*-specific by Southern blotting (data not shown), was approximately 274 nt larger than expected (4). Nucleotide sequencing of the PCR product revealed the normal *HER-2* coding sequence through nucleotide 1171, followed by a 274-nt insertion that was contiguous with exon 9 sequence. Analysis of the predicted protein product shows that the first 340 aa residues, starting with the initiator methionine and signal sequence, are identical to p185 *HER-2* (4) and are followed by a 79-aa extension and a termination codon encoded by the insertion (Fig. 1). Inspection of the 79-aa sequence shows a consensus N-linked glycosylation site (underlined) and a proline content of 19% (Fig. 1). The inserted sequence is designated ECDIIIa because it is located at the boundary between subdomains II and III (23) in the extracellular domain of p185*HER-2*.

Examination of the 5' and 3' junctions of the divergent sequence reveals consensus splice donor and acceptor sites

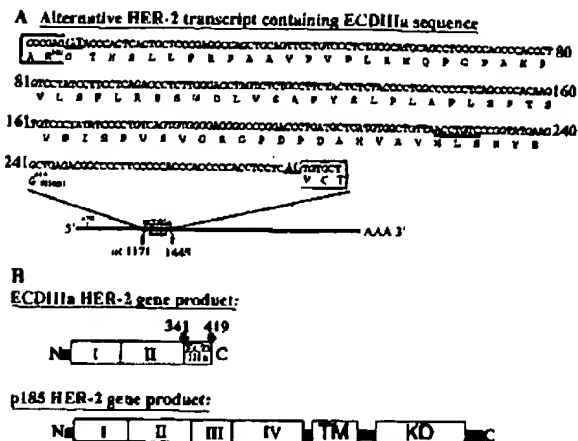


FIG. 1. Nucleotide sequence and deduced amino acid sequence encoded by the 274-nt insertion into *HER-2* mRNA. The *HER-2* ECD coding sequence from exons 1-9 was amplified by PCR from a cDNA library from SKOV-3 cells. A product of ≈ 1,420 bp, found to be *HER-2* specific by Southern blot analysis, was subcloned, and the nucleotide sequence was determined. (A) The nucleotide sequence is shown for the 274-nt insertion (outside the box) and for the adjacent 5' and 3' sequences, enclosed in the box. The insertion is located between nucleotide residues 1171 and 1172 and after amino acid residue 340 in p185*HER-2* by using the numbering of Coussens et al. (4). The consensus 5' and 3' splice sites are underlined and shown in larger print. The inserted sequence is in-frame with 5' *HER-2* exon sequence and is deduced to encode a 79-aa extension after Arg-340 (R³⁴⁰). A consensus asparagine-linked glycosylation site is underlined. Comparison of the inserted nucleotides and their predicted amino acid sequence with sequences in GenBank showed no obvious homologies. (B) The predicted product of the alternative transcript is a truncated secreted protein that contains subdomains I and II identical to p185 and is missing the transmembrane domain and cytoplasmic domain. If fully glycosylated, the expected size is 65-70 kDa. For comparison, the schematic structure of p185 *HER-2* indicates subdomains I, II, III, and IV in the ECD, the transmembrane domain (TM), and the kinase domain (KD).

(24) and includes a pyrimidine tract and potential branchpoint adenine residues near the 3' end of the insert sequence (Fig. 1). PCR analysis of genomic DNA indicates that the 274 nt are contiguous with *HER-2* exonic sequence (J.K.D., J.P.A., and G.M.C., unpublished work) and that the inserted sequence is intron 8 based on the location of intron 8 in the homologous EGFR and *HER-3* genes (25).

Alternative Transcripts Containing the ECDIIIa Sequence Are Expressed in Human Fetal Kidney and Liver. Poly(A)⁺ mRNA from a variety of human fetal tissues, prepared as a Northern blot, was hybridized with a radiolabeled probe specific for the unique ECDIIIa sequence. A 4.8-kb mRNA was detected in kidney as well as in the human embryonic kidney cell line, HEK-293, whereas a 2.6-kb transcript was detected in liver (Fig. 2A). The 4.8-kb transcript likely corresponds to the full-length 4.5-kb transcript with the 274-nt intron sequence, and the 2.6-kb transcript may correspond to the previously described 2.3-kb alternative transcript (5, 22) with the retained intron. When the blot was stripped and hybridized with a probe specific for the 5' *HER-2* coding sequence, a broad band representing the 4.8- and 4.5-kb mRNAs was detected in fetal kidney tissues, and the truncated 2.6-kb transcript was detected in liver, showing that these alternative transcripts contain *HER-2* ECD sequences. Because the inserted ECDIIIa sequence contains a termination codon, both of these alternative transcripts are predicted to encode the same truncated protein product.

A Secreted Protein of ≈ 68 kDa Contains the ECDIIIa Sequence. Antisera against the purified ECDIIIa peptide was

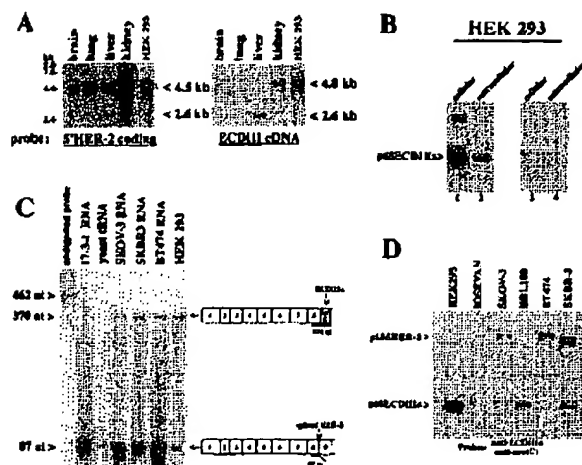


FIG. 2. Expression of the *HER-2* alternative transcript containing the ECDIIIa sequence. (A) Poly(A)⁺ mRNA (2.5 μ g) from different human fetal tissues (CLONTECH) or isolated TriReagent (Molecular Research Center, Cincinnati) from HEK-293 cells was analyzed as a Northern blot as described (36) by using a ³²P-labeled antisense RNA probe complementary to the ECDIIIa sequence. The blot was stripped and reprobed with a ³²P-labeled cDNA probe specific for the 5' *HER-2* exon sequence. (B) Sequence-specific reactivity of anti-ECDIIIa domain antibody with a protein of \sim 68 kDa in HEK-293 cells was detected in cell extract protein (20 μ g) and 20 μ l of media conditioned (CM) by HEK-293 cells. To prepare CM, 15-cm plates of confluent cells were washed 3 \times with PBS and then incubated for 24 hr with 12 ml of serum-free media. The CM was clarified by centrifugation at 13,000 \times g for 20 min and was concentrated by using an Amicon filter that retains proteins of \geq 30,000 daltons. Western blot analysis of cell extract and CM was conducted exactly as described (19). The blot was probed with anti-ECDIIIa diluted 1:10,000 (lanes 1 and 2) or with anti-ECDIIIa diluted 1:10,000 containing 50 μ g/ml of purified His-tagged ECDIIIa peptide (lanes 3 and 4). (C) Ribonuclease protection assay was conducted to detect p68ECDIIIa mRNA and p185HER-2 mRNA. A template for antisense RNA probe synthesis was constructed by PCR amplification using a forward primer that is identical to *HER-2* cDNA sequence at nucleotides 1131–1152 and a reverse primer (5'-GCACGGATCCATAGCAGACTGAGGAGG-3'), which contains a 3' *Bam*HI site and is complementary to the sequence spanning the 3' splice site of the ECDIIIa sequence. The PCR product was digested with *Bam*HI, cloned, and sequenced, and an antisense RNA probe was transcribed by using [α -³²P]CTP and the T7/SP6 Riboprobe Synthesis System (Promega). RNA hybrids were prepared, digested with RNaseA (Boehringer Mannheim) and RNase T1 (Life Technologies, Grand Island, NY), denatured, and electrophoresed in a 5% polyacrylamide/urea gel as described (37). A fragment of 370 nt was protected from RNase digestion, which is the size expected for RNA containing 5' *HER-2* exon sequence and ECDIIIa sequence as illustrated on the right, and was in SKOV-3, SKBR-3, and BT474 RNA, but not in yeast tRNA nor in *HER-2* transfected 17-3-1 cells. A protected fragment of 87 nt, expected for RNA containing 5' exon sequence but not ECDIIIa sequence, was detected in all cell lines but not in tRNA. (D) Extracts (15 μ g of protein) of the indicated cell lines were resolved by SDS/PAGE in 7.5% acrylamide gels and analyzed as a Western blot using both antibodies specific for p68 (anti-ECDIIIa) and for p185 (anti-neu C).

used in a Western blot of protein from HEK-293 cells, which express the ECDIIIa mRNA. A 68-kDa protein in cell extract and in extracellular media reacted with anti-ECDIIIa antibody but not with preimmune sera (data not shown), and reactivity was blocked by preincubation of the antisera with purified ECDIIIa peptide (Fig. 2B). The 68-kDa protein was further characterized as the product of the alternative transcript based on its reactivity with antipeptide antibody against residues 151–165 of p185HER-2 (26). The larger protein of \sim 125 kDa may be an aggregate of p68. The cDNA sequence of the alternative transcript (Fig. 1) predicts a secreted protein

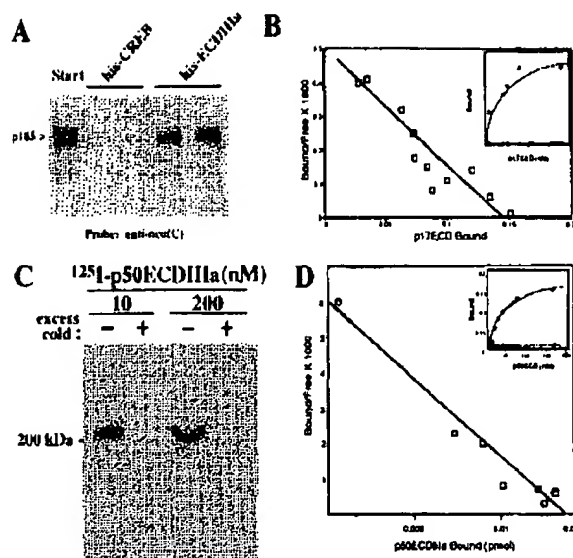


FIG. 3. The ECDIIIa protein specifically associates with p185HER-2. (A) 17-3-1 cell extract (100 μ g) was incubated in duplicate with 50 μ l of packed volume of Ni-nitrilotriacetic acid agarose coupled to 20 μ g of His-tagged ECDIIIa peptide containing the intron-encoded 79 residues or to 20 μ g of His-tagged CREB fragment in 200 μ l of wash buffer (20 mM Tris, pH 8.0/300 mM NaCl) at room temperature for 1 hr with shaking. The resin then was washed four times with 500 μ l of wash buffer, and proteins were eluted by incubation with 50 μ l of SDS-sample buffer at 100°C for 2 min. Eluted proteins were analyzed by Western blot analysis using antibodies against the C terminus of p185, anti-neu(C). (B) Various concentrations of radiolabeled His-tagged ECDIIIa peptide, p17, were incubated with *HER-2*-transfected 17-3-1 cells or parental 3T3 cells. Binding results were analyzed by using the Scatchard method and by plotting the saturation curve (Inset). (C) Radiolabeled p50 was bound to 17-3-1 cells and then incubated with the crosslinking reagent BS³. The washed cells were extracted and immunoprecipitated with 5 μ l of anti-neu(C) as described (26). The immune complex was washed and resolved by SDS/PAGE, and radiolabeled complexes were detected by autoradiography. (D) Various concentrations of radiolabeled p50, purified from bacteria, were incubated with 17-3-1 cells or parental 3T3 cells. Binding results were analyzed by using the Scatchard method and by plotting the saturation curve (Inset).

product of 65–70 kDa if five N-linked glycosylation sites are glycosylated (27).

p68ECDIIIa Expression Is Not Elevated in Proportion to p185HER-2 in Carcinoma Cell Lines in which the *HER-2* Gene Is Amplified. The alternative transcript containing the ECDIIIa sequence could not be detected by Northern analysis of carcinoma cell lines. Therefore the more sensitive ribonuclease protection assay was used by using an antisense probe that spans the entire ECDIIIa sequence and flanking 5' *HER-2* exon sequence. The alternative *HER-2* mRNA with the ECDIIIa insert was detected at less than 5% of the fully spliced transcripts in SKOV-3, SKBR-3, and BT474 cells, which all have their *HER-2* gene amplified about eight times (28), and was expressed at 25–30% of the p185HER-2 transcript in HEK-293 cells (Fig. 2C).

Fig. 2D shows that p185 was enhanced in the carcinoma cell lines that have their *HER-2* gene amplified. However, there was not a corresponding elevation in p68ECDIIIa. In comparison, p185 was expressed at very low levels in the HEK-293, IOSEVAN, and HBL100 nontumorigenic cells, whereas p68 was easily detected. The levels of cellular p68 were reflected in the amount secreted from these cell lines (data not shown). These results suggest that a mechanism may exist to maintain low levels of p68 when p185HER-2 is amplified in carcinoma cells.

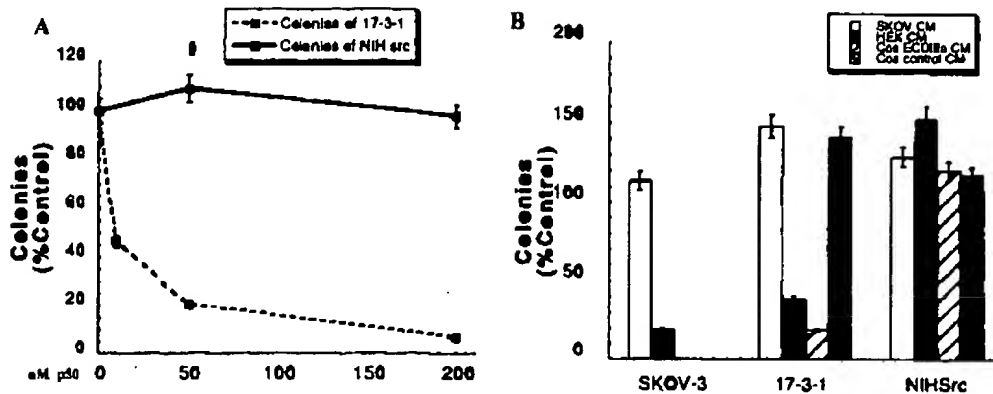


FIG. 5. Anchorage-independent growth of cells in the presence and absence of the ECDIIIa ligand. About 3,000 cells, suspended in media containing 0.3% Difco agar, were plated onto a 0.5-ml layer of media containing 0.5% agar in 12-well plates. Colonies containing at least 50 cells were counted in triplicate wells and expressed as mean percentages of untreated controls at 21 days for SKOV-3 cells and at 14 days for 17-3-1 cells or NIH-src transformed cells. (A) P50ECDIIIa, purified from bacteria, or vehicle were added at the indicated concentrations to the soft agar cultures. (B) Colony formation was in the absence or presence of CM (200 μ g protein) prepared from SKOV-3 cells (SKOV-CM), from HEK-293 cells (HEK-CM), or in 20 \times CM from ECD-transfected or control, vector-transfected Cos-7 cells.

markedly reduced within 10 min of treatment, and then were partially restored by 4 hr (Fig. 4A). The restoration of dimers may occur because the p50ECDIIIa protein is inactivated with time in binding buffer, or p50 may be internalized. A native source of p68ECDIIIa in conditioned media (CM) from HEK-293 cells (see Fig. 2B) as well as the CM of p68ECDIIIa transfected Cos-7 cells compared with the CM from control, vector-transfected Cos-7 cells, also inhibited the levels of p185-containing dimers (Fig. 4A and B). However, neither an irrelevant His-tagged protein purified from bacteria, nor the intron-encoded 79-residue peptide (used in Fig. 3A) inhibited p185 dimers (data not shown).

To examine the impact of p50ECDIIIa on tyrosine phosphorylation, 17-3-1 cells were treated with recombinant p50, crosslinked, and examined as a Western blot that was probed first with antiphosphotyrosine antibody then stripped and probed with anti-p185. There was a dose-dependent decrease in the phosphotyrosine signal associated with p185 that correlated with inhibition of dimers (Fig. 4C). Depression of the tyrosine phosphorylation signal, like dimer inhibition, was transient with restoration to nearly control levels by 6 hr (data not shown). The decreased phosphotyrosine signal was caused, in part, by suppression of the level of tyrosine phosphorylation as well as by a small decrease in the amount of p185HER-2, suggesting that both tyrosine dephosphorylation and down-regulation of p185 occurred in response to p50ECDIIIa.

The Anchorage-Independent Growth of Cells that Overexpress p185HER-2 Is Inhibited in the Presence of the ECDIIIa Protein. Overexpression of p185HER-2 causes transformation of cells, manifested by their anchorage-independent growth (8, 9). To examine the effect of the ECDIIIa protein on anchorage-independent growth, 17-3-1 cells were plated in soft agar cultures containing recombinant p50ECDIIIa. The number of colonies, consisting of at least 50 cells, was inhibited in a dose-dependent manner with about 90% inhibition at 200 nM of p50 (Fig. 5A). Neither the His-tagged CREB protein, nor the recombinant peptide identical to the C terminus of p50ECDIIIa (described in Fig. 3A and B) affected the growth in soft agar (data not shown). The CM from ECDIIIa-transfected Cos-7 cells, but not from control vector-transfected Cos-7 cells, also inhibited the soft agar colony formation by 17-3-1 cells, indicating that the mammalian recombinant ECDIIIa protein displayed the growth inhibitory activity (Fig. 5B). In the presence of the native ligand, p68, from HEK-293 CM, growth in soft agar of 17-3-1 cells and of the SKOV-3 ovarian carcinoma cells, which also depend on p185HER-2

overexpression for anchorage-independent growth (29, 30), was inhibited several-fold compared with cultures treated with CM from the SKOV-3 cells (Fig. 5B and C), which does not contain detectable p68 (data not shown). The growth in soft agar of the NIH 3T3 cells, transformed by the *src* oncogene (*src*⁵²⁷), was not suppressed by any source of the ECDIIIa protein (Fig. 5A and B), indicating that its inhibitory effect was specific for cells transformed by p185HER-2.

DISCUSSION

The results presented here demonstrate tissue-specific expression of alternative *HER-2* mRNA, which contains an additional 274 nt, probably intron 8. The protein product of the alternative transcript, designated herstatin, was secreted from human cells.

Herstatin binds specifically to *HER-2*-transfected cells at nM affinity to a single class of saturable binding sites. A K_D of ≈ 14 nM was determined for the bacterially produced herstatin (Fig. 3D). Binding studies using the glycosylated version, synthesized in mammalian cells, will be necessary to determine the affinity of native herstatin. An extensive search has not yet yielded identification of a characterized, secreted factor that specifically binds with high affinity to p185HER-2 (1–3). Although radiolabeled herstatin was crosslinked to p185HER-2 at the cell surface (Fig. 3C), we cannot rule out the possibility that a coreceptor may be required for binding.

The significance of the C-terminal sequence of herstatin, encoded by the retained intron, was suggested by the specific, high-affinity ($K_D \approx 61$ nM) binding to *HER-2*-transfected cells and by the association with p185HER-2 displayed by the recombinant peptide (Fig. 3A and B). One model is that the intron-encoded sequence confers binding to p185; a possibility generally supported by the *HER-2*-specific binding displayed by the recombinant peptide. Another model, which cannot yet be ruled out, is that the N terminus of herstatin, consisting of subdomains I and II from the extracellular domain of p185, is sufficient for binding to p185 and for the biological activity. The role of the retained intron, in the second model, would be to supply a termination codon to cause truncation, because subdomains I and II, in the context of the entire extracellular domain, do not bind to p185 (31, 32).

Binding of ligands for mammalian EGFR family members is tightly coupled to stimulation of receptor dimerization and tyrosine phosphorylation (1–3, 33). Although herstatin binds with high affinity, it does not activate, but rather inhibits p185

(Fig. 4). Disappearance of dimers within 10 min suggests that herstatin either disrupts existing dimers, shifts the equilibrium between dimers and monomers by stabilizing monomers, or causes their down-regulation and degradation. Herstatin may inhibit dimerization in a dominant negative fashion by occupying monomeric receptors and blocking their recruitment into dimers (32). We also observed a depression in p185-associated tyrosine phosphorylation that correlated with dimer inhibition (Fig. 4C). The significance of the decline in tyrosine phosphorylation to signal transduction will require determination of the specific phosphorylation sites in p185HER-2 that are affected.

Anchorage-independent growth, a property of tumorigenic cells that overexpress *HER-2* (17, 18, 29, 30), was suppressed by bacterial recombinant herstatin, CM from Cos-7 cells transfected with herstatin, and CM from human cells that secrete native herstatin, whereas cells transformed by *src*⁵²⁷ were not inhibited. There are several possible mechanisms by which herstatin could interfere with anchorage-independent growth. Transient interruption of *HER-2* signaling caused by disruption of dimers and inhibition of tyrosine phosphorylation may be sufficient to allow apoptosis to occur in suspended cells. Herstatin also may execute a signal in addition to or distinct from disruption of p185 dimers. Whatever the mechanism, growth inhibition by herstatin could provide a selective pressure for overexpression of p185 to overcome the repressive effects of herstatin in tumor cells with *HER-2* gene amplification (Fig. 2D).

Very few natural ligands that inhibit receptor tyrosine kinases have been identified to date. The Argos protein is an example of an extracellular inhibitor of the *Drosophila* EGFR (34). Likewise, angiopoietin-2 is a natural antagonist for the Tie 2 endothelial receptor tyrosine kinase (35). Herstatin is distinguished from these by its structure, which consists of part of the extracellular domain of the receptor to which it binds.

In summary, our results support the model that herstatin is a naturally occurring inhibitor of p185HER-2. Future studies investigating its impact on growth factor-induced recruitment of p185 into heterodimers and on second messengers that propagate *HER-2* signaling will be required to understand the extent to which herstatin interferes with p185 signaling. As a growth inhibitor (Fig. 5), herstatin could have therapeutic value against human cancers that are driven by overexpression of p185HER-2.

We thank Dr. J. Kaplan for support, Dr. D. Kabat for advice, S. Kozak for help with the binding studies, and B. Lin for technical support. This work was supported by the Department of Defense Breast Cancer Research Program (to G.M.C.) and by the National Institutes of Health (to G.M.C. and J.P.A.). J.K.D. is a predoctoral fellow of the Department of Defense Breast Cancer Research Program. A.J. was supported by a National Institutes of Health grant to Dr. B. Ullman.

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